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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
07898-070001

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)
09/807123

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/05331	9 August 2000	9 August 1999

TITLE OF INVENTION
METHOD OF PRODUCING COPOLYMER POLYESTER

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other documents or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

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I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

April 5, 2001 — Date of Deposit

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Gildardo Vargas, Jr. — Typed Name of Person Signing

U.S. APPLICATION NO. (IF KNOWN) 09/807123	INTERNATIONAL APPLICATION NO. PCT/JP00/05331	ATTORNEY'S DOCKET NUMBER 07898-070001

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)- (5)):

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1000**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$710**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$690**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00
Surcharge of **$130** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$0.00

Claims	Number Filed	Number Extra	Rate	
Total Claims	28 - 20 =	8	x **$18**	$144.00
Independent Claims	1 - 3 =	0	x **$80**	$0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ **$270**	$270.00
TOTAL OF ABOVE CALCULATIONS =				$1,274.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$0.00
SUBTOTAL =				$0.00
Processing fee of **$130** for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))				$0.00
TOTAL NATIONAL FEE =				$0.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$40.00
TOTAL FEES ENCLOSED =				$1,314.00
			Amount to be refunded:	$
			Charged:	$

a. ☒ A check in the amount of $1,314.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 06-1050 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

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SPECIFICATION

Method of Producing Copolymer polyester

FIELD OF THE INVENTION

The present invention relates to a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene, and a NADPH-acetoacetyl CoA reductase gene, and a method of producing copolymer polyester using the transformant.

BACKGROUND OF THE PRESENT INVENTION

BACKGROUND ART

Many microorganisms are known to bio-synthesize poly-3-hydroxybutanoic acid (P(3HB)) and accumulate the fine granular products in its cells as energy storage materials. P(3HB) extracted from microorganisms is a thermoplastic polymer having a melting temperature around 180℃. Currently, P(3HB) is receiving attention as an eco-friendly plastic for environmental conservation because of its good biodegradation ability and biological compatibility. Moreover, P(3HB) can be synthesized from regenerative carbon sources including sugar and vegetable oil using various microorganisms. On the other hand, P(3HB) has a poor impact resistance since it is a high crystallinity polymer. This physical property has hindered the commercialization of P(3HB). However, the impact resistance of P(3HB) can be improved by producing a copolymer of long chain 3-hydroxyalkanoic acid (3HA) units and 3HB unit, so as to produce a flexible material. For example, random copolymer polyester

P(3HB-co-3HV) of 3HB and 3-hydroxyvaleric acid (3HV) with a carbon number of 5, also known as Biopol™, is synthesized by cultivation the bacteria *Ralstonia eutropha* (previously known as *Alcaligenes eutrophus*) in medium supplemented with glucose as a carbon source and propionic acid (European Patent Application No. 0052459, 1981). In addition, a random copolymer P(3HB-co-3HH) of 3HB and 3-hydroxyhexanoic acid (3HH) is synthesized by *Aeromonas caviae*. This copolymer and its production method have been studied and developed as described in Japanese Patent Laid Open Publication Nos. 5-93049 and 7-265065. P(3HB-co-3HH) copolymer has been shown to be a flexible polymer material because its crystallinity decreases as 3HH unit composition increases. Furthermore, P(3HB-co-3HH) copolymer has a good thermostability and mold ability so that it can be processed into a strong string, or into a transparent, flexible film (Y. Doi, S. Kitamura, H. Abe, Macromolecules 28, 4822-4823, 1995).

Pseudomonas sp. strain 61-3 (JCM 10015) is known to contain polyester polymerases including PhaC1 (Japanese Laid Open Publication No. 10-276781, J. Bacteriol., 180, 6459-6467, 1998) and PhaC2 (J. Bacteriol., 180, 6459-6467, 1998), which can use various 3HA units whose carbon number ranges from 4 to 12 as substrates. However, copolymer polyester produced by *Pseudomonas* sp. strain 61-3 is not a preferable plastic material because it becomes amorphous due to its low 3HB composition.

DESCRIPTION OF THE INVENTION

An object of the present invention is to provide a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene, and a NADPH-acetoacetyl CoA reductase gene, and a method of producing a

copolymer polyester with a high composition of 3HB using the transformant.

As a result of thorough studies on the above problems, the present inventors have completed the invention by finding that transformants obtained by transforming *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase 1 gene derived from *Pseudomonas* sp. strain 61-3 (*phaC1* gene), a β-ketothiolase gene (*phbA* gene) derived from *Ralstonia eutropha*, and a NADPH-acetoacetyl CoA reductase gene (*phbB* gene) derived from *Ralstonia eutropha*, produce a P(3HB-co-3HA) having molar composition of 3HB ranges from 80 to 95% and having 3HA units with a carbon number of 4 to 12.

In other words, the present invention provides a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, β-ketothiolase gene, and NADPH-acetoacetyl CoA reductase gene.

The present invention provides a transformant wherein the polyester polymerase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO: 2 or 4, or

(b) a protein having an amino acid sequence including deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 2 or 4, and having polyester polymerase activity.

Further, the present invention provides a transformant wherein the polyester polymerase gene comprises the following DNA (a) or (b):

(a) a DNA containing a nucleotide sequence represented by SEQ ID NO: 1 or 3, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID

NO: 1 or 3 under stringent conditions, and encoding a protein with polyester polymerase activity.

Further, the present invention provides a transformant wherein the β-ketothiolase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO: 6, or

(b) a protein having an amino acid sequence including deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO:6, and having β-ketothiolase gene activity.

Further, the present invention provides a transformant wherein the β-ketothiolase gene comprises the following DNA (a) or (b):

(a) a DNA having an nucleotide sequence represented by SEQ ID NO: 5, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 5 under stringent conditions and encoding a protein with β-ketothiolase activity.

Further, the present invention provides a transformant wherein the NADPH-acetoacetyl CoA reductase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO:8, or

(b) a protein having an amino acid sequence including deletion, substitution or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 8, and having NADPH-acetoacetyl CoA reductase activity.

Furthermore the present invention provides a transformant wherein the NADPH-acetoacetyl CoA reductase gene comprises the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 7, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 7 under stringent conditions, and encoding a protein with NADPH-acetoacetyl CoA

reductase activity.

Furthermore, the present invention provides a transformant which is a bacterium belonging to the genus *Pseudomonas* or *Ralstonia*. An example of the bacterium belonging to the genus *Pseudomonas* is the genus *Pseudomonas* sp. strain 61-3.

The present invention also provides a method of producing copolymer polyester which comprises the steps of culturing a transformant, and collecting polyester (for example, a polyester comprising 3-hydroxyalkanoic acid units with a carbon number of 4 to 12) from the culture product. An example of the polyester produced herein comprises 3-hydroxyalkanoic acid units with a carbon number of 4 to 12 wherein molar composition of 3-hydroxybutanoic acid is 80 to 95% molar fraction.

Detailed description of this invention will now be given as follows.

1. A Host for Transformation

Hosts that can be used for transformation are not specifically limited so far as they can express each gene contained in a recombinant vector. Examples of the host include bacteria belonging to the genus *Pseudomonas*, such as *Pseudomonas putida*, *Pseudomonas* sp. strain 61-3, those belonging to the genus *Ralstonia*, such as *Ralstonia eutropha*, those belonging to the genus *Bacillus*, such as *Bacillus subtilis*, those belonging to the genus *Escherichia*, such as *Escherichia coli*, yeast belonging to the genus *Saccharomyces*, such as *Saccharomyces cerevisiae*, yeast belonging to the genus *Candida*, such as *Candida maltosa*, and animal cells, such as COS cells and CHO cells.

In particular, a cell whose certain naturally occuring polyester polymerase gene is disrupted can be used to allow the host cell to produce a polyester having a desired composition. Figure 1 shows the synthetic pathway of polyhydroxybutanoic acid

(P(3HB)), and of a copolymer (P(3HB-co-3HA)) of 3-hydroxybutanoic acid and 3-hydroxyalkanoic acid.

The *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, can be constructed by deleting the terminus of a polyhydroxybutanoic acid polymerase gene to cause mutation in the gene, and introducing the mutated gene into *Pseudomonas* sp. strain 61-3 to cause homologous recombination between the mutated polyhydroxybutanoic acid polymerase gene and a polyhydroxybutanoic acid polymerase gene on a chromosome. Whether the polyhydroxybutanoic acid polymerase gene is disrupted or not can be confirmed by Southern hybridization using a part of the gene as a probe. That is, it can be confirmed by examining if a band of hybridization shifts to an expected position relative to a band of a wild type.

2. Recombinant Vector

Recombinant vectors of this invention can be obtained by ligating (inserting) a polyester polymerase gene, β-ketothiolase gene, and NADPH-acetoacetyl CoA reductase gene into an appropriate expression vector.

Examples of the polyester polymerase gene include a *phaC1* gene or a *phaC2* gene derived from *Pseudomonas* sp. strain 61-3. A nucleotide sequence of the *phaC1* gene is shown in SEQ ID NO: 1, and an amino acid sequence of the polyester polymerase encoded by the *phaC1* gene is shown in SEQ ID NO: 2. Mutations including deletion, substitution, and addition of one or more amino acids may occur in the amino acid sequence so far as proteins having these amino acid sequences have polyester polymerase activity. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 2 or 4; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be added to the amino acid sequence represented by 2 or 4; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence

represented by SEQ ID NO: 2 or 4. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 1 or 3 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having polyester polymerase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and a sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

An example of a β-ketothiolase gene is a *phbA* gene derived from *Ralstonia eutropha*. A nucleotide sequence of the *phbA* gene is shown in SEQ ID NO:5, and an amino acid sequence of the β-ketothiolase encoded by the *phbA* gene is shown in SEQ ID NO:6. So far as proteins having these amino acid sequences possess β-ketothiolase activity, mutations including deletion, substitution, and addition of one or more amino acids may occur in these amino acid sequences. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 6; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be added to the amino acid sequence represented by SEQ ID NO: 6; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence represented by SEQ ID NO: 6. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 5 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having β-ketothioesterase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

An example of a NADPH-acetoacetyl CoA reductase gene is a *phbB* gene derived from *Ralstonia eutropha*. A nucleotide sequence of the *phbB* gene is shown in SEQ ID NO:7, and an amino acid sequence of the NADPH-acetoacetyl CoA reductase encoded by the *phbB* gene is shown in SEQ ID NO: 8. So far as proteins having these amino acid sequences possess NADPH-acetoacetyl CoA reductase activity, mutations

including deletion, substitution, and addition of one or more amino acids may occur in these amino acid sequences. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 8; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be added to the amino acid sequence represented by SEQ ID NO: 8; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence represented by SEQ ID NO: 8. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 7 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having NADPH-acetoacetyl CoA reductase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

Vectors used herein to insert each gene as described above are not specifically limited so far as they can autonomously replicate in hosts. Examples of the vector include plasmid DNA and phage DNA. When *Escherichia coli* is used as a host cell, examples of vectors include plasmid DNAs, such as pBR322, pUC18, and pBluescript II, phage DNAs, such as EMBL3, M13, and λgt11. When yeast is used as a host cell, examples of vectors include YEp13 and YCp50; when an animal cell is used as a host cell, examples of vectors include pcDNAI, and pcDNAI/Amp (Invitrogen). In addition, when bacteria belonging to the genus *Ralstonia* and those belonging to the genus *Pseudomonas* are used as host cells, examples of vectors include pLA2917 (ATCC37355) having RK2 replication origin, and pJRD215 (ATCC 37533) having RSF1010 replication origin. These replication origins are replicated and retained in a broad range of hosts.

A gene can be inserted into a vector by integrating a DNA fragment with the above gene into a vector DNA fragment digested with a restriction enzyme. At this time, the above gene must be inserted into a vector so that the gene function is exhibited.

Particularly, gene expression requires insertion of a gene downstream of a promoter. Any promoter can be used so far as it can express in a host. When *Escherichia coli* is used as a host cell, examples of promoters include trp promoter, lac promoter, PL promoter, PR promoter, and T7 promoter; when yeast is used as a host cell, examples of promoters include gal1 promoter and gal 10 promoter.

When bacteria belonging to the genus *Pseudomonas* are used as host cells, a promoter region of upstream of a $phaC1_{Ps}$ gene or a $phbCAB_{Re}$ gene may be used as a promoter. A nucleotide sequence of upstream of the $phaC1_{Ps}$ gene is as shown in SEQ ID NO: 9, and that of upstream of the $phbCAB_{Re}$ operon is as shown in SEQ ID NO: 10.

If necessary, a terminator, enhancer, splicing signal, polyA additional signal, selection marker, and ribosome binding sequence (SD) and the like may be integrated into the vector of this invention. Examples of the selection marker include ampicillin-, tetracycline-, neomycin-, kanamycin, and chroramphenicol-resistant genes. Particularly when bacteria belonging to the genus *Pseudomonas* are used as host cells, a terminator region of downstream of a $phbCAB_{Re}$ operon may be used as a terminator. A nucleotide sequence downstream of the $phbCAB_{Re}$ operon is as shown in SEQ ID NO: 11.

3. Preparation of Transformants

The transformant of this invention can be obtained by introducing a recombinant vector obtained in 2 above into a host cell of 1 above. Examples of a method of introducing a recombinant DNA into a bacterium include a method using calcium ions (Current Protocols in Molecular Biology, vol. 1, p.1.8.1, 1994) or electroporation (Current Protocols in Molecular Biology, vol. 1, p.1.8.4, 1994). A plasmid can be introduced into a bacterium belonging to the genus *Pseudomonas* by the conjugation transfer method (Friedrich et al. : J. Bacteriol. 147: 198-205, 1981).

Examples of a method of introducing a recombinant DNA into yeast include electroporation (Methods. Enzymol., 194:182-187, 1990), the spheroplast method (Proc. Natl. Acad. Sci., USA, 84:1929-1933 (1978)), the lithium acetate method (J. Bacteriol., 153:163-168 , 1983). Examples of a method of introducing a recombinant DNA into an animal cell include electroporation and the calcium phosphate method.

The transformant (strain) *Pseudomonas* sp. BB49 obtained by transforming *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a plasmid pJBB49-*phb*, and the transformant *Pseudomonas* sp. KSc46 obtained by transforming with a plasmid pJKSc46-*pha*, and the transformant *Pseudomonas* sp. KSc54 obtained by transforming with a plasmid pJKSc54-*phb* were deposited with National Institute of Bioscience and Human-Technology, National Institute of Advanced Industrial Science and Technology (1-1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 5, 1999. The accession numbers received were FERM BP-7263, FERM BP-7264 and FERM BP-7265, respectively.

4. Production of Polyester

Polyester is produced by culturing the transformants of this invention in media, allowing them to synthesize and accumulate copolymer polyester in the culture cells or culture products, and collecting the polyester from the culture cells or culture products. The transformants of this invention are cultured in media by standard methods employed for culturing hosts.

Examples of culture media for culturing transformants obtained using bacteria belonging to the genus *Ralstonia* or the genus *Pseudomonas* as host cells include media containing carbon sources assimilable by microorganisms, and containing a limited amount of any one of nitrogen sources, inorganic salts, or other organic nutrient sources.

For example, a medium used herein contains nitrogen sources limited to 0.01% to 0.1%. Culture is performed at 25 to 37℃ aerobically for 2 to 7 days to allow the cells to accumulate polyester within the cells, followed by collection of the polyester.

Examples of carbon sources include carbohydrates such as glucose, fructose, sucrose, and maltose. Oil and fat-related substances with a carbon number of 4 or more than 4 may be used as carbon sources. Examples of such carbon sources include natural oil, such as corn oil, soy oil, safflower oil, sunflower oil, olive oil, palm oil, colza oil, fish oil, whale oil, pig oil, or cattle oil; fatty acids, such as butanoic acid, pentanoic acid, hexanoic acid, octanoic acid, decanoic acid, lauric acid, oleic acid, palmitin acid, linoleic acid, linolic acid or myristic acid, or esters of these fatty acids; octanol, lauryl alcohol, oleyl alcohol, or palmityl alcohol, or esters of these alcohols.

Examples of nitrogen sources include ammonia, ammonium salts such as ammonium chloride, ammonium sulfate, and ammonium phosphate in addition to peptone, meat extract, yeast extract, corn steep liquor. Examples of inorganic substances include potassium phosphate, potassium secondary phosphate, magnesium phosphate, magnesium sulfate, sodium chloride.

Normally, shake-culture is performed under aerobic conditions at 25 to 37℃ for 24 hours or more following induction of expression. Antibiotics including kanamycin, ampicillin, and tetracycline may be added to media while culturing.

When a transformant (microorganisms) transformed by an expression vector containing an inducible promoter is cultured, an inducer may be added to the medium. For example, isopropyl-β-D-thiogalactoside (IPTG), indoleacrylic acid (IAA) and the like may be added to the media.

Examples of media for culturing transformants obtained using animal

cells as host cells include RPMI-1640 media, DMEM media, or those supplemented with fetal calf serum. Culturing is usually performed in the presence of 5% CO_2 at 30 to 37℃ for 14 to 28 days. Antibiotics including kanamycin and penicillin may be added to the media.

Polyester of this invention can be purified as follows. Transformants are collected by centrifugation from culture solution, washed with distilled water, and then dried. Next, the dried transformants are suspended in chloroform and heated to extract polyester. Residue is removed by filtration. Then methanol is added to the chloroform solution to precipitate polyester. Following removal of supernatant by filtration and centrifugation, the product is dried to obtain purified polyester. The resulting polyester can be used as materials for biodegradable strings, films, and various containers. Whether the resulting polyester is of interest or not is confirmed by standard techniques including gas chromatography and nuclear magnetic resonance methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a synthetic pathway of polyester.

Figs. 2A – 2F show steps to construct a recombinant vector.

Fig. 3 shows a structure of the recombinant vector used for transformation of *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*).

Fig. 4 shows the results of tensile test on copolymer polyester film obtained by this invention.

EXAMPLES

A detailed description of the present invention will be given as follows.

However, the technical scope of the present invention is not limited by the examples.

Example 1 Establishment of a polyhydroxybutanoic acid polymerase gene-disruptant of *Pseudomonas* sp. strain 61-3

Pseudomonas sp. strain 61-3 (JCM 10015) contains polyhydroxybutanoic acid polymerase (PhbC) in addition to polyhydroxyalkanoic acid polymerase (PhaCl and PhaC2) that can use a broad range of substrates with a carbon number of 4 to 12. Therefore, this strain 61-3 synthesizes a blend of polyester (P(3HB)) having a sole unit of 3-hydroxybutanoic acid and copolymer polyester (P(3HB-co-3HA)) containing 3HA units with a carbon number of 4 to 12. A 3HB unit is often favored in comparison to PhaC1 or PhaC2 as a substrate by PhbC, which has a high affinity with the 3HB unit, so that copolymer polyester with a high 3HB molar composition is not synthesized. Accordingly, a polyhydroxybutanoic acid polymerase gene-disruptant of *Pseudomonas* sp. strain 61-3 was established.

First, the 5' terminal region of 342 bp and the 3' terminal region of 418 bp were deleted from a gene (*phbC*$_{Ps}$ gene) encoding polyhydroxybutanoic acid polymerase of *Pseudomonas* sp. strain 61-3, thereby preparing a deleted polyhydroxybutanoic acid polymerase gene fragment (*Eco*RI-*Pst*I fragment) of 941 bp. Next, the resulting *Eco*RI-*Pst*I fragment was ligated to *Eco*RI and *Pst*I sites of pBR322, thereby constructing a plasmid pBREP9 (Tc^r) for disrupting polyhydroxybutanoic acid polymerase gene. The obtained plasmid pBREP9 (Tc^r) was introduced into *Pseudomonas* sp. strain 61-3 suspended in 8 mM HEPES buffer (pH 7.2) containing 272 mM sucrose by electroporation (conditions: 7.5 kV/cm, 800 Ω, 25 μF). *phbC*$_{Ps}$ gene-disrupted strain (*phbC*::*tet*) was screened for strains capable of growing on LB media containing tetracyclin. Then chromosomal DNAs prepared from some of the screened strains and wild strains were digested with appropriate restriction enzymes. Following digestion, Southern hybridization with a part of *phbC*$_{Ps}$ gene as a probe was performed, thereby selecting and obtaining strains showing a band that had shifted to a

position of an expected molecular weight.

Example 2 Construction of a Recombinant Vector

Figure 2 shows steps to construct a recombinant vector. Cleavage and ligation of DNA fragments were performed according to standard techniques (Sambrook et al. : Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory Press, 1989). As shown in Fig. 2, pBSEX22 and pGEM'-phbCAB were used as start plasmids. Here, pBSEX22 was constructed by inserting a 2.2-kb *Eco*RI-*Xba*I region containing *phaC1* gene of *Pseudomonas* sp. strain 61-3 into pBluescript II KS+. pGEM'-phbCAB was constructed by amplifying *phbCAB* gene of *Ralstonia eutropha* H16 (ATCC 17699) (J. Biol. Chem., 264, 15293-15297, 1989; J. Biol. Chem., 264, 15298-15303, 1989) by PCR and inserting the resulting PCR fragment into a pGEM-T (Promega) vector having disrupted *Nde*I and *Pst*I sites.

At the final step of the construction of a recombinant vector, the DNA fragment encoding a gene of interest was inserted and ligated into a plasmid pJRD215 (ATCC37533) capable of replicating within *Pseudomonas* sp. strain 61-3. In other words, a plasmid pJASc22 was constructed by cutting out a *Apa*I-*Sac*I fragment which contains polyester polymerase 1 gene ($phaC1_{Ps}$ gene) derived from *Pseudomonas* sp. strain 61-3 and a promoter (P_{Ps} promoter) of this gene from pBSEX22, and by inserting and ligating the fragment into *Apa*I and *Sac*I sites of pJRD215. Further, a plasmid pJBB49-*phb* was constructed by inserting and ligating *Bam*HI-*Bam*HI fragment which contains $phbCAB_{Re}$ operon promoter (P_{Re} promoter) derived from *Ralstonia eutropha*, $phaC1_{Ps}$ gene, β-ketothiolase gene ($phbA_{Re}$ gene) derived from *Ralstonia eutropha*, NADPH-acetoacetyl CoA reductase gene ($phbB_{Re}$ gene) derived from *Ralstonia eutropha*, and $phbCAB_{Re}$ operon terminator (T_{Re} terminator) derived from *Ralstonia eutropha* into a *Bam*HI site of pJRD215. Furthermore, a plasmid pJKSc46-*pha* was constructed by inserting and ligating *Kpn*I-*Sac*I fragment, which contains P_{Ps} promoter, $phaC1_{Ps}$ gene, $phbA_{Re}$ gene, $phbB_{Re}$ gene and T_{Re} terminator into *Kpn*I and *Sac*I sites of

pJRD215. Moreover, a plasmid pJKSc54-*phab* was constructed by inserting and ligating *Kpn*I-*Sac*I fragment, which contains P_{Ps} promoter, *phaC1*$_{Ps}$ gene, P_{Re} promoter, *phbA*$_{Re}$ gene, *phbB*$_{Re}$ gene and T_{Re} terminator, into *Kpn*I and *Sac*I sites of pJRD215. Structures of the four types of plasmids obtained are shown in Fig. 3.

Example 3 Construction of a *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) Transformant

A transformant was constructed by introducing the plasmid obtained in Example 2 into the *phbC*$_{Ps}$ gene-disruptant (*phbC*::*tet*) of *Pseudomonas* sp. strain 61-3 obtained in Example 1 by the conjugation transfer method. That is, four types of plasmids pJASc22, pJBB49-*phb*, pJKSc46-*pha*, and pJKSc54-*phab* were separately transformed into *E.coli* strain S17-1 by the calcium chloride method. Next, the obtained transformants and *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) were separately cultured overnight in 1.5 ml of LB medium at 37℃ and 28℃, respectively. Subsequently, 0.1 ml of the culture product of *E.coli* and 0.1 ml of that of *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) were mixed and cultured for 4 hours at 28℃. After culturing, the mixture was plated over MS agar media (0.9% sodium diphosphate, 0.15% monopotassium phosphate, 0.05% ammonium chloride, 2% glucose, 0.1% (v/v) Trace element solution ($CoCl_2 \cdot 6H_2O$ 0.218g, $FeCl_3$ 9.7g, $CaCl_2$ 7.8g, $NiCl_3 \cdot 6H_2O$ 0.118g, $CrCl_3 \cdot 6H_2O$ 0.105g, and $CuSO_4 \cdot 5H_2O$ 0.156g dissolved in 1 liter of 0.1N hydrochloric acid), 1.5% agar, 50mg/l kanamycin, 12.5 g/l tetracyclin) and cultured for 2 to 5 days at 28℃.

Transformants were obtained by isolating colonies that had grown on the MS agar medium. Four types of transformants having pJASc22, pJBB49-*phb*, pJKSc46-*pha*, and pJKSc54-*phab* were designated as *Pseudomonas* sp. strain ASc22, BB49, KSc46, and Ksc54, respectively.

Example 4 Polyester Synthesis by *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) Transformants

Polyester was produced by transformants obtained in Example 3. First, each

strain of *Pseudomonas* sp. 61-3 (*phbC::tet*), ASc22, BB49, KSc46, and KSc54 was inoculated in 100 ml of MS medium containing 2% glucose, and then cultured in Sakaguchi flasks (shaking flasks) for 48 hours at 28℃. Cells were collected by centrifugation, washed with distilled water, and freeze-dried. The dried cells were measured for weight, polyester content, and polyester composition.

That is, 2 ml of a mixture of sulfuric acid – methanol (15:85) and 2 ml of chloroform were added to 10 to 30 mg of the dried cells, then the containers were tightly stoppered. Then, methyl ester was obtained by decomposing intracellular polyester by heating for 140 minutes at 100℃. Then 1ml of distilled water was added to the methyl ester, followed by vigorous stirring. Next the mixture was allowed to stand to separate into two layers. The organic layer of the lower layer was taken out and the composition was analyzed by capillary gas chromatography. A gas chromatograph used herein was GC-17A (manufactured by SHIMADZU CORPORATION) and capillary column was NEUTRA BOND-1 (manufactured by GL Science, column length 25 m, column internal diameter 0.25 mm, liquid film thickness 0.4 μm). Temperature was raised at a rate of 8℃/min from the initial temperature of 100℃. Table 1 shows the results.

Table 1 Copolymer polyester production by *Pseudomonas* sp. strain 61-3 (*phbC::tet*)

Strain	Dry cell weight (g/l)	Polyester content (wt %)	Polyester composition (mol%)					
			3HB (C_4)	3HHx (C_6)	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})	3H5DD (C_{12})
phbC::tet	0.7	4	36	0	6	23	20	15
Asc22	0.7	6	64	0	2	15	11	8
BB49	1.7	38	92	0	1	4	2	1
Ksc46	2.6	37	81	0	1	9	5	4
Ksc54	2.5	45	92	0	1	3	3	1

Note) 3HB: 3-hydroxybutanoic acid; 3HHx: 3-hydroxyhexanoic acid; 3HO: 3-hydroxyoctanoic acid, 3HD: 3-hydroxydecanoic acid; 3HDD: 3-hydroxydodecanoic

acid, 3H5DD: 3-hydroxy-cis-5-dodecanoic acid

As clearly shown in Table 1, each type of recombinant cell achieved relatively efficient accumulation of 5 to 50 wt % polyester using 2 % glucose as a carbon source. *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) produced copolymer polyester P(3HB-co-3HA) having a 3HB molar composition of 36 mol%, while the strain ASc22 with the introduced *phaC1* gene produced polyester having a 3HB molar composition of approximately 64 mol %, slightly higher than that of the former polyester. On the other hand, the strains BB49, KSc46, and KSc54, into which *phbA* and *phbB* genes derived from *Ralstonia eutropha* had been introduced, produced polyester having as high as 81 to 92 mol % of a 3HB molar composition. Moreover, these strains accumulated polyester in the cells at a high intracellular accumulation ratio of 37 to 45 wt %. Unlike P(3HB), copolymer polyester P(3HB-co-3HA) with a high 3HB molar composition has better flexibility and good impact resistance. Therefore, it is concluded that more practical biodegradable plastic can be produced at higher efficiency by using the above described strains.

Example 5 Mechanical strength of copolymer polyester

Mechanical strength of the copolymer polyester with a high 3HB molar composition obtained in this invention was examined. First, a film with a thickness of approximately 150 μm was prepared using copolymer polyester P(3HB-co-3HA) consisting of 94 mol % 3-hydroxybutanoic acid (carbon number: 4) and 6 mol% 3-hydroxyalkanoic acid (carbon number: 6 to 12). Then a tensile test was performed on the film at a tensile strength of 100 mm/min and at room temperature according to the method of Doi, Y. et al. (Macromolecules 28: 4822, 1995). Figure 4 shows the result. Tensile strength, Young's modulus, and elongation to break calculated based on a curve as shown in Fig. 4 were 17 MPa, 0.22Gpa, and 680%, respectively. These values are equivalent to those of low density polyethylene, which is made from general petroleum (film grade; 10 MPa, 0.17 GPa, and 620%). In addition, known

biodegradable copolymer polyesters P(3HB-co-10% 4HB) (Saito, Y. et al. : Int. J. Biol. Macromol., 16: 99, 1994) and P(3HB-co-10% 3HHx) (Doi, Y. et al. : Macromolecules 28: 4822, 1995) have a tensile strength of 24 MPa and 21MPa, and fracture elongation percentage of 242% and 400%, respectively. Thus, the biodegradable copolymer polyester obtained in this invention is confirmed to have flexibility superior to known biodegradable polyesters.

As described above, biodegradable plastic with mechanical strength equivalent to that of petroleum plastic, and high flexibility that known types of degradable plastic lack was able to be produced according to the present invention.

INDUSTRIAL APPLICABILITY

A method of this invention is to synthesize copolymer polyester P(3HB-co-3HA) comprising 3-hydroxyalkanoic acid units with a carbon number of 4 to 12 and having a high 3HB molar composition. Such a polyester is useful because it has good thermal stability and mold ability, and can be made into biodegradable plastic with impact resistance better than that of P(3HB).

CLAIMS

1. A transformant obtainable by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene and a NADPH-acetoacetyl CoA reductase gene.

2. The transformant of claim 1 wherein the polyester polymerase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO: 2 or 4, or

(b) a protein having an amino acid sequence including deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 2 or 4, and having polyester polymerase activity.

3. The transformant of claim 1 wherein the polyester polymerase gene comprises the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 1 or 3, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 1 or 3 under stringent conditions, and encoding a protein with polyester polymerase activity.

4. The transformant of claim 1 wherein the β-ketothiolase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO:6, or

(b) a protein having an amino acid sequence including deletion, substitution or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 6, and having β-ketothiolase activity.

5. The transformant of claim 1 wherein the β-ketothiolase gene comprises the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 5, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 5 under stringent conditions, and encoding a protein with β-ketothiolase activity.

6. The transformant of claim 1, wherein the NADPH-acetoacetyl CoA reductase gene comprises a DNA encoding the following protein (a) or (b):
(a) a protein having an amino acid sequence represented by SEQ ID NO:8, or
(b) a protein having an amino acid sequence including deletion, substitution or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 8, and having NADPH-acetoacetyl CoA reductase activity.

7. The transformant of claim 1, wherein the NADPH-acetoacetyl CoA reductase gene comprises the following DNA (a) or (b):
(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 7, or
(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 7 under stringent conditions, and encoding a protein with NADPH-acetoacetyl CoA reductase activity.

8. The transformant of claim 1 which is a bacterium belonging to the genus *Pseudomonas* or the genus *Ralstonia*.

9. The transformant of claim 8, wherein the bacterium belonging to the genus *Pseudomonas* is *Pseudomonas* sp. strain 61-3 (JCM10015).

10. A method of producing copolymer polyester which comprises the steps of culturing the transformant of any one of claims 1 to 9, and collecting polyester from the culture product.

11. The method of producing copolymer polyester of claim 10, wherein the polyester comprises 3-hydroxyalkanoic acid units with a carbon number of 4 to 12.

12. The method of producing copolymer polyester of claim 11, wherein the 3-hydroxyalkanoic acid units contain 3-hydroxybutanoic acid with 80 to 95% molar fraction.

ABSTRACT

A transformant, whose polyhydroxybutanoic acid polymerase gene is disrupted, having a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene, and a NADPH-acetoacetyl CoA reductase gene.

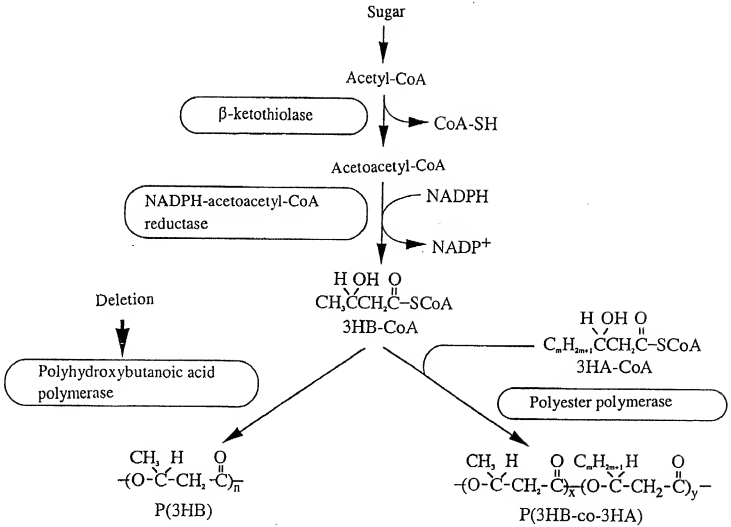
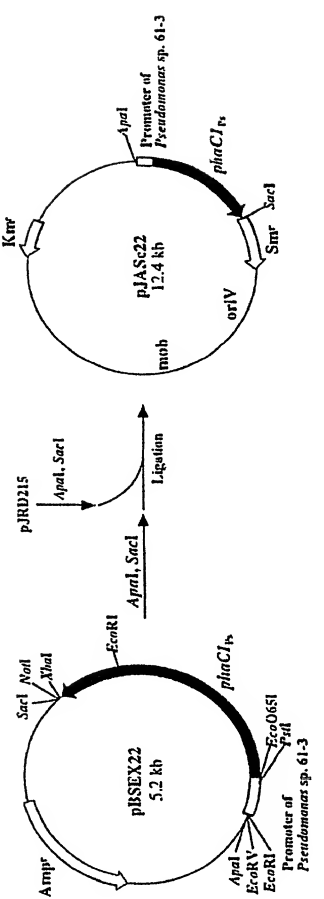


FIG. 1

FIG. 2A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Ampr
SacI
NotI
XbaI
EcoRI
ApaI
EcoRV
EcoRI
Promoter of
Pseudomonas sp. 61-3
phaCIPs
Eco0651
PstI
ApaI, SacI
Ligation
pJRD215
ApaI, SacI
pJASc22
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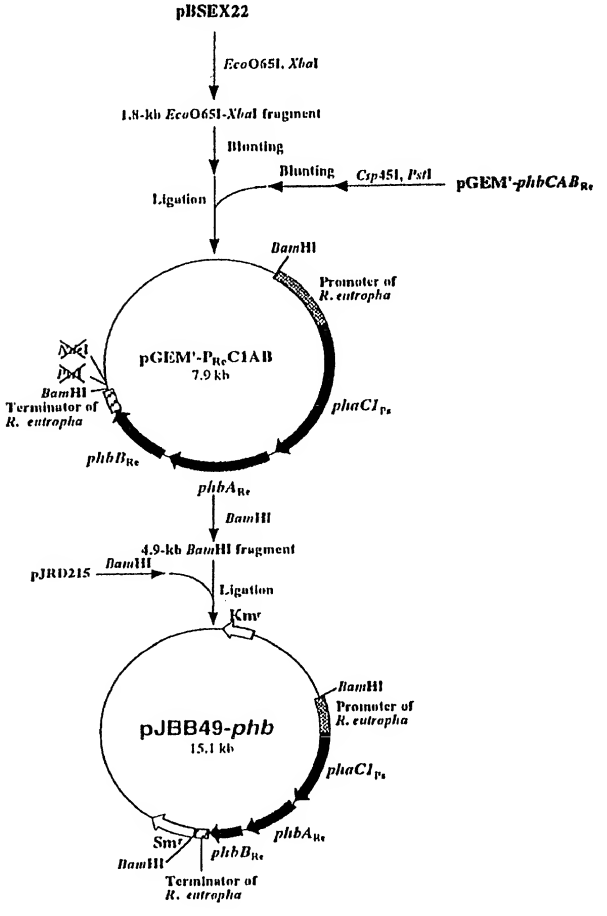
ApaI
Promoter of
Pseudomonas sp. 61-3
phaCIPs
SacI
Smr
oriV
mob

FIG. 2 B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Blunting
Blunting
Csp45I, PstI
pGEM'-phbCAB$_{Re}$
Ligation
BamHI
Promoter of R. eutropha
pGEM'-P$_{Re}$C1AB
7.9 kb
BamHI
Terminator of R. eutropha
phaC1$_{Ps}$
phbB$_{Re}$
phbA$_{Re}$
BamHI
4.9-kb BamHI fragment
pJRD215
BamHI
Ligation
Km^r
BamHI
Promoter of R. eutropha
pJBB49-phb
15.1 kb
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phbA$_{Re}$
Sm^r
phbB$_{Re}$
BamHI
Terminator of R. eutropha

FIG. 2C

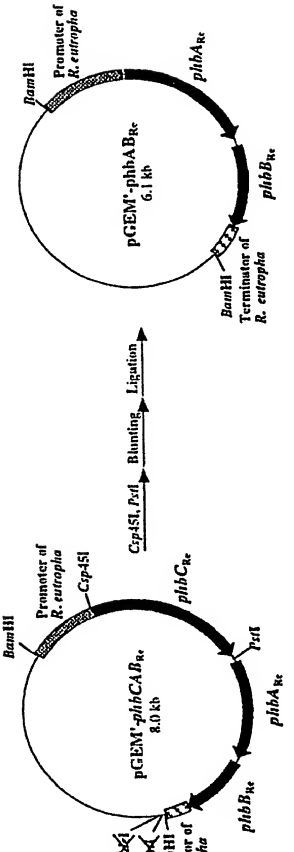
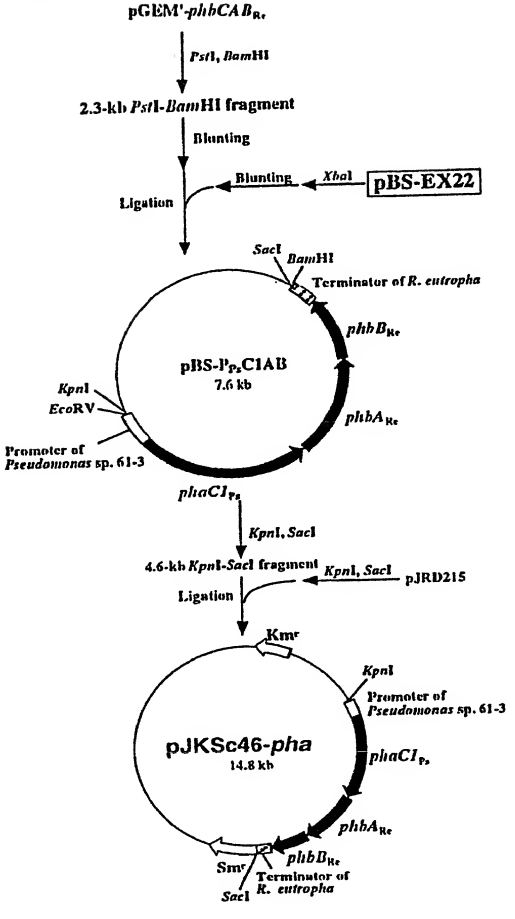

BamHI
Promoter of R. eutropha
phbA_Re
pGEM'-phbAB_Re 6.1 kb
phbB_Re
BamHI Terminator of R. eutropha
Csp45I, PstI
Blunting
Ligation
BamHI
Promoter of R. eutropha
Csp45I
phbC_Re
pGEM'-phbCAB_Re 8.0 kb
PstI
phbA_Re
phbB_Re
BamHI Terminator of R. eutropha

FIG. 2D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PstI, BamHI
2.3-kb PstI-BamHI fragment
Blunting
Blunting
XbaI
pBS-EX22
Ligation
SacI
BamHI
Terminator of R. eutropha
phbB$_{Re}$
pBS-P$_{Ps}$C1AB
7.6 kb
phbA$_{Re}$
KpnI
EcoRV
Promoter of Pseudomonas sp. 61-3
phaC1$_{Ps}$
KpnI, SacI
4.6-kb KpnI-SacI fragment
KpnI, SacI
pJRD215
Ligation
Km^r
KpnI
Promoter of Pseudomonas sp. 61-3
pJKSc46-pha
14.8 kb
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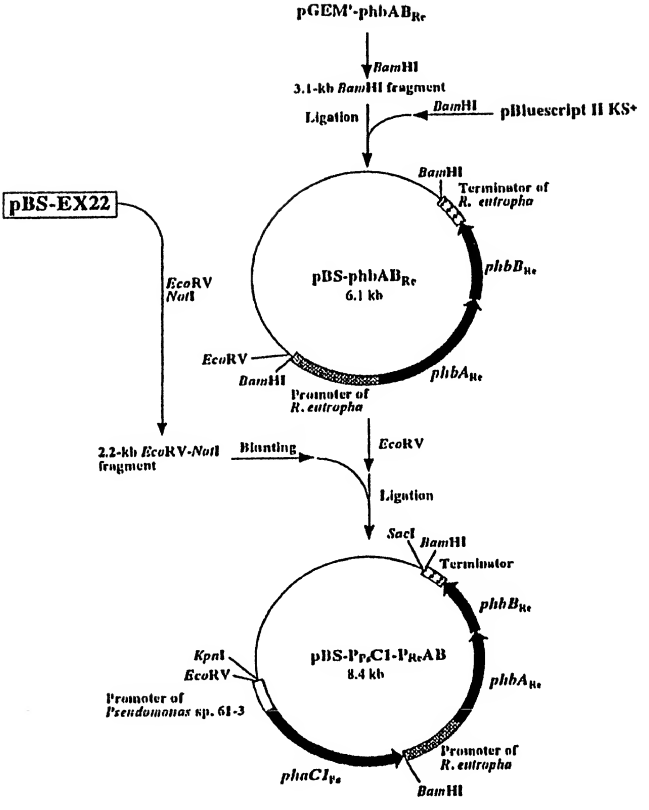
phbB$_{Re}$
Sm^r
Terminator of R. eutropha
SacI

FIG. 2 E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BamHI
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Terminator of R. eutropha
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EcoRV
NotI
pBS-phbAB$_{Re}$
6.1 kb
phbB$_{Re}$
EcoRV
BamHI
phbA$_{Re}$
Promoter of R. eutropha
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EcoRV
Ligation
SacI
BamHI
Terminator
phbB$_{Re}$
KpnI
EcoRV
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Promoter of R. eutropha
phaC1$_{Pa}$
BamHI

FIG. 2 F

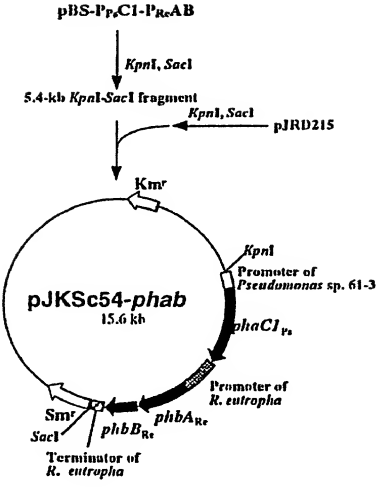

pBS-P$_{Ps}$C1-P$_{Re}$AB
KpnI, SacI
5.4-kb KpnI-SacI fragment
KpnI, SacI
pJRD215
Km^r
KpnI
Promoter of
Pseudomonas sp. 61-3
pJKSc54-phab
15.6 kb
phaC1$_{Ps}$
Promoter of
R. eutropha
phbA$_{Re}$
Sm^r
SacI
phbB$_{Re}$
Terminator of
R. eutropha

FIG. 3

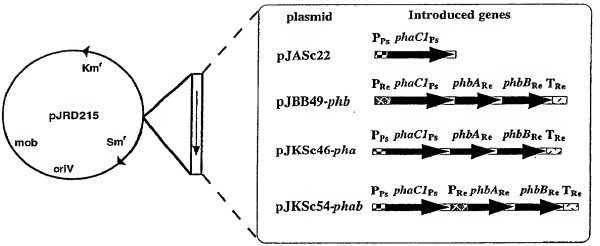
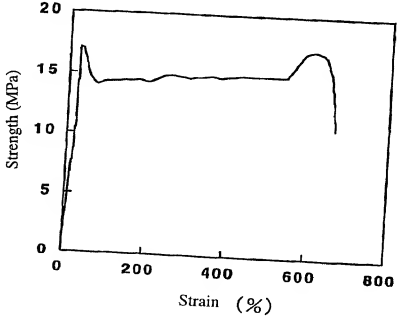

plasmid
Introduced genes
pJASc22
P_{Ps} *phaC1*$_{Ps}$
pJBB49-*phb*
P_{Re} *phaC1*$_{Ps}$ *phbA*$_{Re}$ *phbB*$_{Re}$ T_{Re}
pJKSc46-*pha*
P_{Ps} *phaC1*$_{Ps}$ *phbA*$_{Re}$ *phbB*$_{Re}$ T_{Re}
pJKSc54-*phab*
P_{Ps} *phaC1*$_{Ps}$ P_{Re} *phbA*$_{Re}$ *phbB*$_{Re}$ T_{Re}
pJRD215
Km^r
Sm^r
mob
oriV

FIG. 4



Attorney's Docket No.:_____

DECLARATION, POWER OF ATTORNEY AND PETITION

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Method of producing copolymer polyester

the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____ as Application Serial No. _____ and amended on _____.
- ■ was filed as PCT international application Number PCT/JP00/05331 on August 9, 2000, and was amended under PCT Article 19 on _____ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
225102/1999	Japan	August 9, 1999	■ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

_____ _____

(Application Number) (Filing Date)

_____ _____

(Application Number) (Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

(19)

And I (We) hereby appoint: Richard J. Anderson, Registration No. 36, 732; Gregory P. Einhorn, Registration No. 38,440; Mark S. Ellinger, Registration No. 34,812; J. Eldora L. Ellison, Registration No. 39,967; Peter Fasse, Registration No. 32,983; J. Patrick Finn, III, Registration No. 44,109; Janis K. Fraser, Registration No. 34,819; John W. Freeman, Registration No. 29,066; Diane L. Gardner, Registration No. 36,518; Scott C. Harris, Registration No. 32,030; John F. Hayden, Registration No. 37,640; George Heibel, Registration No. 42,648; Donald C. Kordich, Registration No. 38,213; Ronald C. Lundquist, Registration No. 37,875; Anita L. Meiklejohn, Registration No. 35,283; Mike P. Reed, Registration No. 45, 647; Reginald Suyat, Registration No. 28, 172; Y. Rocky Tsao, Registration No. 34, 054; Hans R. Troesch, Registration No. 36, 950; and Dorothy P. Whelan, Registration No. 33,814.

I(We) hereby request that all correspondence regarding this application be sent to the firm of FISH & RICHARDSON P.C. whose Post office address is: 4350 La Jolla Village Drive, Suite 500, San Diego CA 92122, U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of Inventor

March 15, 2001

Date

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Hiromi Matsusaki

Signature of Inventor

March 15, 2001

Date

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Citizen of: Japan

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Sakae-cho, Asaka-shi, Saitama
351-0012 Japan

NAME OF THIRD JOINT INVENTOR

Signature of Inventor

Date

Residence:

Citizen of:

Post Office Address:

配列表

SEQUENCE LISTING

<110> Riken ; Japan Science and Technology Corporation

<120> The method of production of coplymerized polyester

<130> PH-1046-PCT

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            20                  25                  30

act tct gcc cga atg gtt tta acc caa gcc atc aaa caa ccc att cac   144
Thr Ser Ala Arg Met Val Leu Thr Gln Ala Ile Lys Gln Pro Ile His
        35                  40                  45

agc gtc aag cac gtc gcg cat ttt ggc atc gag ctg aag aac gtg atg   192
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Ser Val Lys His Val Ala His Phe Gly Ile Glu Leu Lys Asn Val Met

50 55 60

ttt ggc aaa tcg aag ctg caa ccg gaa agc gat gac cgt cgt ttc aac 240

Phe Gly Lys Ser Lys Leu Gln Pro Glu Ser Asp Asp Arg Arg Phe Asn

65 70 75 80

gac ccc gcc tgg agt cag aac cca ctc tac aaa cgt tat cta caa acc 288

Asp Pro Ala Trp Ser Gln Asn Pro Leu Tyr Lys Arg Tyr Leu Gln Thr

85 90 95

tac ctg gcg tgg cgc aag gaa ctc cac gac tgg atc ggc aac agc aaa 336

Tyr Leu Ala Trp Arg Lys Glu Leu His Asp Trp Ile Gly Asn Ser Lys

100 105 110

ctg tcc gaa cag gac atc aat cgc gct cac ttc gtg atc acc ctg atg 384

Leu Ser Glu Gln Asp Ile Asn Arg Ala His Phe Val Ile Thr Leu Met

115 120 125

acc gaa gcc atg gcc ccg acc aac agt gcg gcc aat ccg gcg gcg gtc 432

Thr Glu Ala Met Ala Pro Thr Asn Ser Ala Ala Asn Pro Ala Ala Val

130 135 140

aaa cgc ttc ttc gaa acc ggc ggt aaa agc ctg ctc gac ggc ctc aca 480

Lys Arg Phe Phe Glu Thr Gly Gly Lys Ser Leu Leu Asp Gly Leu Thr

145 150 155 160

cat ctg gcc aag gac ctg gta aac aac ggc ggc atg ccg agc cag gtg 528

His Leu Ala Lys Asp Leu Val Asn Asn Gly Gly Met Pro Ser Gln Val

165 170 175

gac atg ggc gct ttc gaa gtc ggc aag agt ctg ggg acg act gaa ggt 576

Asp Met Gly Ala Phe Glu Val Gly Lys Ser Leu Gly Thr Thr Glu Gly

180 185 190

gca gtg gtt ttc cgc aac gac gtc ctc gaa ttg atc cag tac cgg ccg 624

Ala Val Val Phe Arg Asn Asp Val Leu Glu Leu Ile Gln Tyr Arg Pro

195 200 205

```
acc acc gaa cag gtg cat gag cga ccg ctg ctg gtg gtc cca ccg cag   672
Thr Thr Glu Gln Val His Glu Arg Pro Leu Leu Val Val Pro Pro Gln
    210                 215                 220
atc aac aag ttt tat gtg ttt gac ctg agc ccg gat aaa agc ctg gcg   720
Ile Asn Lys Phe Tyr Val Phe Asp Leu Ser Pro Asp Lys Ser Leu Ala
225                 230                 235                 240
cgc ttc tgc ctg agc aac aac cag caa acc ttt atc gtc agc tgg cgc   768
Arg Phe Cys Leu Ser Asn Asn Gln Gln Thr Phe Ile Val Ser Trp Arg
                245                 250                 255
aac ccg acc aag gcc cag cgt gag tgg ggt ctg tcg act tac atc gat   816
Asn Pro Thr Lys Ala Gln Arg Glu Trp Gly Leu Ser Thr Tyr Ile Asp
            260                 265                 270
gcg ctc aaa gaa gcc gtc gac gta gtt tcc gcc atc acc ggc agc aaa   864
Ala Leu Lys Glu Ala Val Asp Val Val Ser Ala Ile Thr Gly Ser Lys
        275                 280                 285
gac atc aac atg ctc ggc gcc tgc tcc ggt ggc att acc tgc acc gcg   912
Asp Ile Asn Met Leu Gly Ala Cys Ser Gly Gly Ile Thr Cys Thr Ala
    290                 295                 300
ctg ctg ggt cac tac gcc gct ctc ggc gag aag aag gtc aat gcc ctg   960
Leu Leu Gly His Tyr Ala Ala Leu Gly Glu Lys Lys Val Asn Ala Leu
305                 310                 315                 320
acc ctt ttg gtc agc gtg ctc gac acc acc ctc gac tcc cag gtt gca  1008
Thr Leu Leu Val Ser Val Leu Asp Thr Thr Leu Asp Ser Gln Val Ala
                325                 330                 335
ctg ttc gtc gat gag aaa acc ctg gaa gct gcc aag cgt cac tcg tat  1056
Leu Phe Val Asp Glu Lys Thr Leu Glu Ala Ala Lys Arg His Ser Tyr
            340                 345                 350
cag gcc ggc gtg ctg gaa ggc cgc gac atg gcc aaa gtc ttc gcc tgg  1104
Gln Ala Gly Val Leu Glu Gly Arg Asp Met Ala Lys Val Phe Ala Trp
```

```
        355                 360                 365
atg cgc cct aac gac ctg atc tgg aac tac tgg gtc aac aac tac ctg   1152
Met Arg Pro Asn Asp Leu Ile Trp Asn Tyr Trp Val Asn Asn Tyr Leu
    370                 375                 380
ctg ggt aac gag cca ccg gtc ttc gac att ctt ttc tgg aac aac gac   1200
Leu Gly Asn Glu Pro Pro Val Phe Asp Ile Leu Phe Trp Asn Asn Asp
385                 390                 395                 400
acc acc cgg ttg cct gct gcg ttc cac ggc gat ctg atc gaa atg ttc   1248
Thr Thr Arg Leu Pro Ala Ala Phe His Gly Asp Leu Ile Glu Met Phe
                405                 410                 415
aaa aat aac cca ctg gtg cgc gcc aat gca ctc gaa gtg agc ggc acg   1296
Lys Asn Asn Pro Leu Val Arg Ala Asn Ala Leu Glu Val Ser Gly Thr
            420                 425                 430
ccg atc gac ctc aaa cag gtc act gcc gac atc tac tcc ctg gcc ggc   1344
Pro Ile Asp Leu Lys Gln Val Thr Ala Asp Ile Tyr Ser Leu Ala Gly
        435                 440                 445
acc aac gat cac atc acg ccc tgg aag tct tgc tac aag tcg gcg caa   1392
Thr Asn Asp His Ile Thr Pro Trp Lys Ser Cys Tyr Lys Ser Ala Gln
    450                 455                 460
ctg ttc ggt ggc aag gtc gaa ttc gtg ctg tcc agc agt ggg cat atc   1440
Leu Phe Gly Gly Lys Val Glu Phe Val Leu Ser Ser Ser Gly His Ile
465                 470                 475                 480
cag agc att ctg aac ccg ccg ggc aat ccg aaa tca cgt tac atg acc   1488
Gln Ser Ile Leu Asn Pro Pro Gly Asn Pro Lys Ser Arg Tyr Met Thr
                485                 490                 495
agc acc gac atg cca gcc acc gcc aac gag tgg caa gaa aac tca acc   1536
Ser Thr Asp Met Pro Ala Thr Ala Asn Glu Trp Gln Glu Asn Ser Thr
            500                 505                 510
aag cac acc gac tcc tgg tgg ctg cac tgg cag gcc tgg cag gcc gag   1584
```

```
Lys His Thr Asp Ser Trp Trp Leu His Trp Gln Ala Trp Gln Ala Glu
        515                 520                 525
cgc tcg ggc aaa ctg aaa aag tcc ccg acc agc ctg ggc aac aag gcc   1632
Arg Ser Gly Lys Leu Lys Lys Ser Pro Thr Ser Leu Gly Asn Lys Ala
    530                 535                 540
tat ccg tca gga gaa gcc gcg ccg ggc acg tat gtg cat gaa cgt taa   1680
Tyr Pro Ser Gly Glu Ala Ala Pro Gly Thr Tyr Val His Glu Arg
545                 550                 555
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<210> 2

<211> 559

<212> PRT

<213> Pseudomonas sp. strain 61-3

<400> 2

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Met Ser Asn Lys Asn Ser Asp Asp Leu Asn Arg Gln Ala Ser Glu Asn
  1               5                  10                  15
Thr Leu Gly Leu Asn Pro Val Ile Gly Leu Arg Gly Lys Asp Leu Leu
             20                  25                  30
Thr Ser Ala Arg Met Val Leu Thr Gln Ala Ile Lys Gln Pro Ile His
         35                  40                  45
Ser Val Lys His Val Ala His Phe Gly Ile Glu Leu Lys Asn Val Met
     50                  55                  60
Phe Gly Lys Ser Lys Leu Gln Pro Glu Ser Asp Asp Arg Arg Phe Asn
 65                  70                  75                  80
Asp Pro Ala Trp Ser Gln Asn Pro Leu Tyr Lys Arg Tyr Leu Gln Thr
                 85                  90                  95
Tyr Leu Ala Trp Arg Lys Glu Leu His Asp Trp Ile Gly Asn Ser Lys
            100                 105                 110
```

Leu Ser Glu Gln Asp Ile Asn Arg Ala His Phe Val Ile Thr Leu Met
115 120 125
Thr Glu Ala Met Ala Pro Thr Asn Ser Ala Ala Asn Pro Ala Ala Val
130 135 140
Lys Arg Phe Phe Glu Thr Gly Gly Lys Ser Leu Leu Asp Gly Leu Thr
145 150 155 160
His Leu Ala Lys Asp Leu Val Asn Asn Gly Gly Met Pro Ser Gln Val
165 170 175
Asp Met Gly Ala Phe Glu Val Gly Lys Ser Leu Gly Thr Thr Glu Gly
180 185 190
Ala Val Val Phe Arg Asn Asp Val Leu Glu Leu Ile Gln Tyr Arg Pro
195 200 205
Thr Thr Glu Gln Val His Glu Arg Pro Leu Leu Val Val Pro Pro Gln
210 215 220
Ile Asn Lys Phe Tyr Val Phe Asp Leu Ser Pro Asp Lys Ser Leu Ala
225 230 235 240
Arg Phe Cys Leu Ser Asn Asn Gln Gln Thr Phe Ile Val Ser Trp Arg
245 250 255
Asn Pro Thr Lys Ala Gln Arg Glu Trp Gly Leu Ser Thr Tyr Ile Asp
260 265 270
Ala Leu Lys Glu Ala Val Asp Val Val Ser Ala Ile Thr Gly Ser Lys
275 280 285
Asp Ile Asn Met Leu Gly Ala Cys Ser Gly Gly Ile Thr Cys Thr Ala
290 295 300
Leu Leu Gly His Tyr Ala Ala Leu Gly Glu Lys Lys Val Asn Ala Leu
305 310 315 320
Thr Leu Leu Val Ser Val Leu Asp Thr Thr Leu Asp Ser Gln Val Ala
325 330 335
Leu Phe Val Asp Glu Lys Thr Leu Glu Ala Ala Lys Arg His Ser Tyr

340 345 350

Gln Ala Gly Val Leu Glu Gly Arg Asp Met Ala Lys Val Phe Ala Trp

355 360 365

Met Arg Pro Asn Asp Leu Ile Trp Asn Tyr Trp Val Asn Asn Tyr Leu

370 375 380

Leu Gly Asn Glu Pro Pro Val Phe Asp Ile Leu Phe Trp Asn Asn Asp

385 390 395 400

Thr Thr Arg Leu Pro Ala Ala Phe His Gly Asp Leu Ile Glu Met Phe

405 410 415

Lys Asn Asn Pro Leu Val Arg Ala Asn Ala Leu Glu Val Ser Gly Thr

420 425 430

Pro Ile Asp Leu Lys Gln Val Thr Ala Asp Ile Tyr Ser Leu Ala Gly

435 440 445

Thr Asn Asp His Ile Thr Pro Trp Lys Ser Cys Tyr Lys Ser Ala Gln

450 455 460

Leu Phe Gly Gly Lys Val Glu Phe Val Leu Ser Ser Ser Gly His Ile

465 470 475 480

Gln Ser Ile Leu Asn Pro Pro Gly Asn Pro Lys Ser Arg Tyr Met Thr

485 490 495

Ser Thr Asp Met Pro Ala Thr Ala Asn Glu Trp Gln Glu Asn Ser Thr

500 505 510

Lys His Thr Asp Ser Trp Trp Leu His Trp Gln Ala Trp Gln Ala Glu

515 520 525

Arg Ser Gly Lys Leu Lys Lys Ser Pro Thr Ser Leu Gly Asn Lys Ala

530 535 540

Tyr Pro Ser Gly Glu Ala Ala Pro Gly Thr Tyr Val His Glu Arg

545 550 555

<210> 3

<211> 1683

<212> DNA

<213> Pseudomonas sp. strain 61-3

<220>

<221> CDS

<222> (1)..(1680)

<400> 3

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atg aga gag aaa cca acg ccg ggc ttg ctg ccc aca ccc gcg acg ttc   48
Met Arg Glu Lys Pro Thr Pro Gly Leu Leu Pro Thr Pro Ala Thr Phe
  1               5                  10                  15

atc aac gct cag agt gcg att acc ggt ctg cgc ggc cgg gat ctg ttc   96
Ile Asn Ala Gln Ser Ala Ile Thr Gly Leu Arg Gly Arg Asp Leu Phe
            20                  25                  30

tcg acc ctg cgc agc gtg gcc gcc cac ggc ctg cgt cac ccg gtg cgc  144
Ser Thr Leu Arg Ser Val Ala Ala His Gly Leu Arg His Pro Val Arg
        35                  40                  45

agc gcc cgt cat gtt ctg gca ctg ggc ggc cag ttg ggc cgc gtg ctg  192
Ser Ala Arg His Val Leu Ala Leu Gly Gly Gln Leu Gly Arg Val Leu
    50                  55                  60

ctg ggc gaa acg ctg cac acg ccg aac ccg aaa gac aat cgc ttt gcg  240
Leu Gly Glu Thr Leu His Thr Pro Asn Pro Lys Asp Asn Arg Phe Ala
65                  70                  75                  80

gac ccg acc tgg aga ctg aat ccg ttt tac cgg cgc agc ctg cag gcc  288
Asp Pro Thr Trp Arg Leu Asn Pro Phe Tyr Arg Arg Ser Leu Gln Ala
                85                  90                  95

tat ctg agc tgg cag aaa cag gtc aaa agc tgg atc gat gaa agc ggc  336
Tyr Leu Ser Trp Gln Lys Gln Val Lys Ser Trp Ile Asp Glu Ser Gly
            100                 105                 110
```

atg agt gac gat gac cgc gcc cgc gcg cat ttc gtc ttc gca ctg ctc 384
Met Ser Asp Asp Asp Arg Ala Arg Ala His Phe Val Phe Ala Leu Leu
115 120 125
aat gac gcc gtg tcc ccc tcc aat acc ctg ctc aac ccg cta gcg atc 432
Asn Asp Ala Val Ser Pro Ser Asn Thr Leu Leu Asn Pro Leu Ala Ile
130 135 140
aag gag ctg ttc aac tcc ggt ggc aac agc ctg gtc cgc ggt ctc agc 480
Lys Glu Leu Phe Asn Ser Gly Gly Asn Ser Leu Val Arg Gly Leu Ser
145 150 155 160
cat tta ttc gac gac ctg atg cac aac aac ggg ctg ccc agt cag gtc 528
His Leu Phe Asp Asp Leu Met His Asn Asn Gly Leu Pro Ser Gln Val
165 170 175
acc aaa cac gcc ttc gag att ggc aag acc gtg gca acc acc gcc ggg 576
Thr Lys His Ala Phe Glu Ile Gly Lys Thr Val Ala Thr Thr Ala Gly
180 185 190
tcc gtg gtg ttt cgc aac gag ctg ctc gag ctg atg cag tac aag ccg 624
Ser Val Val Phe Arg Asn Glu Leu Leu Glu Leu Met Gln Tyr Lys Pro
195 200 205
atg agc gaa aaa cag tac gcc aag ccg ttg ctg atc gtc ccg ccg cag 672
Met Ser Glu Lys Gln Tyr Ala Lys Pro Leu Leu Ile Val Pro Pro Gln
210 215 220
att aac aag tac tac att ttc gac ctc agc ccg ggt aac agc ttc gtc 720
Ile Asn Lys Tyr Tyr Ile Phe Asp Leu Ser Pro Gly Asn Ser Phe Val
225 230 235 240
cag tac gca ttg aag aat ggt ctg cag gtg ttc gtg gtc agc tgg cgt 768
Gln Tyr Ala Leu Lys Asn Gly Leu Gln Val Phe Val Val Ser Trp Arg
245 250 255
aac ccg gat gtt cgc cac cgc gaa tgg ggc ctg tcc agt tac gtt gag 816
Asn Pro Asp Val Arg His Arg Glu Trp Gly Leu Ser Ser Tyr Val Glu

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            260                 265                 270
gca ctg gaa gaa gca ctg aat gtt tgc cgc gct atc acc ggc gcg cgc   864
Ala Leu Glu Glu Ala Leu Asn Val Cys Arg Ala Ile Thr Gly Ala Arg
        275                 280                 285
gac gtc aat ctg atg ggc gcc tgt gct ggc ggc ctg acc atc gcg gct   912
Asp Val Asn Leu Met Gly Ala Cys Ala Gly Gly Leu Thr Ile Ala Ala
    290                 295                 300
ctg caa ggt cat ctg caa gcc aag cgg caa ctg cgg cgg gtc tcc agc   960
Leu Gln Gly His Leu Gln Ala Lys Arg Gln Leu Arg Arg Val Ser Ser
305                 310                 315                 320
gcc agc tac ctg gtc agc ctg ctg gat agc cag ata gac agc ccg gcg  1008
Ala Ser Tyr Leu Val Ser Leu Leu Asp Ser Gln Ile Asp Ser Pro Ala
                325                 330                 335
acg ttg ttc gcc gat gag cag acg ctg gaa gcc gcc aag cgc cat tcc  1056
Thr Leu Phe Ala Asp Glu Gln Thr Leu Glu Ala Ala Lys Arg His Ser
            340                 345                 350
tat caa cga ggt gtg ctc gag ggg cgc gac atg gcg aaa atc ttc gcc  1104
Tyr Gln Arg Gly Val Leu Glu Gly Arg Asp Met Ala Lys Ile Phe Ala
        355                 360                 365
tgg atg cgc ccc aat gac ctg atc tgg aac tac tgg gtc aac aac tac  1152
Trp Met Arg Pro Asn Asp Leu Ile Trp Asn Tyr Trp Val Asn Asn Tyr
    370                 375                 380
ctg ctg ggc aaa gaa ccg ccg gcc ttc gac att ctg tat tgg aac agt  1200
Leu Leu Gly Lys Glu Pro Pro Ala Phe Asp Ile Leu Tyr Trp Asn Ser
385                 390                 395                 400
gac aac acg cgc ctg cca gcg gca ttc cat ggc gac ctg ctg gac ttc  1248
Asp Asn Thr Arg Leu Pro Ala Ala Phe His Gly Asp Leu Leu Asp Phe
                405                 410                 415
ttc aag cac aat ccg ctg act cac ccc ggc ggg ctg gag gtc tgt ggc  1296
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```
Phe Lys His Asn Pro Leu Thr His Pro Gly Gly Leu Glu Val Cys Gly
            420                 425                 430
acg cct atc gat ttg cag aag gtc aac gta gac agc ttc agc gtg gcc   1344
Thr Pro Ile Asp Leu Gln Lys Val Asn Val Asp Ser Phe Ser Val Ala
        435                 440                 445
ggc atc aac gac cac atc act ccg tgg gac gcg gtg tac cgc tcg acc   1392
Gly Ile Asn Asp His Ile Thr Pro Trp Asp Ala Val Tyr Arg Ser Thr
    450                 455                 460
ctg ctg ctg ggt ggc gac cgg cgc ttc gta ctg tcc aac agc ggg cat   1440
Leu Leu Leu Gly Gly Asp Arg Arg Phe Val Leu Ser Asn Ser Gly His
465                 470                 475                 480
atc cag agc atc ctc aac ccg ccg agc aac ccc aag tcc aac tac atc   1488
Ile Gln Ser Ile Leu Asn Pro Pro Ser Asn Pro Lys Ser Asn Tyr Ile
                485                 490                 495
gag aac ccc aag ctc agt ggc gat cca cgc gcc tgg tat tac gac ggc   1536
Glu Asn Pro Lys Leu Ser Gly Asp Pro Arg Ala Trp Tyr Tyr Asp Gly
            500                 505                 510
acc cat gtc gaa ggt agc tgg tgg cca cgt tgg ctg agc tgg att cag   1584
Thr His Val Glu Gly Ser Trp Trp Pro Arg Trp Leu Ser Trp Ile Gln
        515                 520                 525
gag cgc tcc ggt acc caa cgc gaa acc ctg atg gcc ctt ggt aac cag   1632
Glu Arg Ser Gly Thr Gln Arg Glu Thr Leu Met Ala Leu Gly Asn Gln
    530                 535                 540
aac tat cca ccg atg gag gcg gcg cca ggt acc tac gtg cgc gtg cgc   1680
Asn Tyr Pro Pro Met Glu Ala Ala Pro Gly Thr Tyr Val Arg Val Arg
545                 550                 555                 560
tga                                                             1683
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<210> 4

<211> 560

<212> PRT

<213> Pseudomonas sp. strain 61-3

<400> 4

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Met Arg Glu Lys Pro Thr Pro Gly Leu Leu Pro Thr Pro Ala Thr Phe
  1               5                  10                  15
Ile Asn Ala Gln Ser Ala Ile Thr Gly Leu Arg Gly Arg Asp Leu Phe
            20                  25                  30
Ser Thr Leu Arg Ser Val Ala Ala His Gly Leu Arg His Pro Val Arg
        35                  40                  45
Ser Ala Arg His Val Leu Ala Leu Gly Gly Gln Leu Gly Arg Val Leu
    50                  55                  60
Leu Gly Glu Thr Leu His Thr Pro Asn Pro Lys Asp Asn Arg Phe Ala
65                  70                  75                  80
Asp Pro Thr Trp Arg Leu Asn Pro Phe Tyr Arg Arg Ser Leu Gln Ala
                85                  90                  95
Tyr Leu Ser Trp Gln Lys Gln Val Lys Ser Trp Ile Asp Glu Ser Gly
            100                 105                 110
Met Ser Asp Asp Asp Arg Ala Arg Ala His Phe Val Phe Ala Leu Leu
        115                 120                 125
Asn Asp Ala Val Ser Pro Ser Asn Thr Leu Leu Asn Pro Leu Ala Ile
    130                 135                 140
Lys Glu Leu Phe Asn Ser Gly Gly Asn Ser Leu Val Arg Gly Leu Ser
145                 150                 155                 160
His Leu Phe Asp Asp Leu Met His Asn Asn Gly Leu Pro Ser Gln Val
                165                 170                 175
Thr Lys His Ala Phe Glu Ile Gly Lys Thr Val Ala Thr Thr Ala Gly
            180                 185                 190
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Ser Val Val Phe Arg Asn Glu Leu Leu Glu Leu Met Gln Tyr Lys Pro
195 200 205
Met Ser Glu Lys Gln Tyr Ala Lys Pro Leu Leu Ile Val Pro Pro Gln
210 215 220
Ile Asn Lys Tyr Tyr Ile Phe Asp Leu Ser Pro Gly Asn Ser Phe Val
225 230 235 240
Gln Tyr Ala Leu Lys Asn Gly Leu Gln Val Phe Val Val Ser Trp Arg
245 250 255
Asn Pro Asp Val Arg His Arg Glu Trp Gly Leu Ser Ser Tyr Val Glu
260 265 270
Ala Leu Glu Glu Ala Leu Asn Val Cys Arg Ala Ile Thr Gly Ala Arg
275 280 285
Asp Val Asn Leu Met Gly Ala Cys Ala Gly Gly Leu Thr Ile Ala Ala
290 295 300
Leu Gln Gly His Leu Gln Ala Lys Arg Gln Leu Arg Arg Val Ser Ser
305 310 315 320
Ala Ser Tyr Leu Val Ser Leu Leu Asp Ser Gln Ile Asp Ser Pro Ala
325 330 335
Thr Leu Phe Ala Asp Glu Gln Thr Leu Glu Ala Ala Lys Arg His Ser
340 345 350
Tyr Gln Arg Gly Val Leu Glu Gly Arg Asp Met Ala Lys Ile Phe Ala
355 360 365
Trp Met Arg Pro Asn Asp Leu Ile Trp Asn Tyr Trp Val Asn Asn Tyr
370 375 380
Leu Leu Gly Lys Glu Pro Pro Ala Phe Asp Ile Leu Tyr Trp Asn Ser
385 390 395 400
Asp Asn Thr Arg Leu Pro Ala Ala Phe His Gly Asp Leu Leu Asp Phe
405 410 415
Phe Lys His Asn Pro Leu Thr His Pro Gly Gly Leu Glu Val Cys Gly

420 425 430

Thr Pro Ile Asp Leu Gln Lys Val Asn Val Asp Ser Phe Ser Val Ala

435 440 445

Gly Ile Asn Asp His Ile Thr Pro Trp Asp Ala Val Tyr Arg Ser Thr

450 455 460

Leu Leu Leu Gly Gly Asp Arg Arg Phe Val Leu Ser Asn Ser Gly His

465 470 475 480

Ile Gln Ser Ile Leu Asn Pro Pro Ser Asn Pro Lys Ser Asn Tyr Ile

485 490 495

Glu Asn Pro Lys Leu Ser Gly Asp Pro Arg Ala Trp Tyr Tyr Asp Gly

500 505 510

Thr His Val Glu Gly Ser Trp Trp Pro Arg Trp Leu Ser Trp Ile Gln

515 520 525

Glu Arg Ser Gly Thr Gln Arg Glu Thr Leu Met Ala Leu Gly Asn Gln

530 535 540

Asn Tyr Pro Pro Met Glu Ala Ala Pro Gly Thr Tyr Val Arg Val Arg

545 550 555 560

<210> 5

<211> 1179

<212> DNA

<213> Ralstonia eutropha

<220>

<221> CDS

<222> (1)..(1179)

<400> 5

atg act gac gtt gtc atc gta tcc gcc gcc cgc acc gcg gtc ggc aag 48

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Met Thr Asp Val Val Ile Val Ser Ala Ala Arg Thr Ala Val Gly Lys
  1               5                  10                  15
ttt ggc ggc tcg ctg gcc aag atc ccg gca ccg gaa ctg ggt gcc gtg   96
Phe Gly Gly Ser Leu Ala Lys Ile Pro Ala Pro Glu Leu Gly Ala Val
            20                  25                  30
gtc atc aag gcc gcg ctg gag cgc gcc ggc gtc aag ccg gag cag gtg   144
Val Ile Lys Ala Ala Leu Glu Arg Ala Gly Val Lys Pro Glu Gln Val
        35                  40                  45
agc gaa gtc atc atg ggc cag gtg ctg acc gcc ggt tcg ggc cag aac   192
Ser Glu Val Ile Met Gly Gln Val Leu Thr Ala Gly Ser Gly Gln Asn
    50                  55                  60
ccc gca cgc cag gcc gcg atc aag gcc ggc ctg ccg gcg atg gtg ccg   240
Pro Ala Arg Gln Ala Ala Ile Lys Ala Gly Leu Pro Ala Met Val Pro
65                  70                  75                  80
gcc atg acc atc aac aag gtg tgc ggc tcg ggc ctg aag gcc gtg atg   288
Ala Met Thr Ile Asn Lys Val Cys Gly Ser Gly Leu Lys Ala Val Met
                85                  90                  95
ctg gcc gcc aac gcg atc atg gcg ggc gac gcc gag atc gtg gtg gcc   336
Leu Ala Ala Asn Ala Ile Met Ala Gly Asp Ala Glu Ile Val Val Ala
            100                 105                 110
ggc ggc cag gaa aac atg agc gcc gcc ccg cac gtg ctg ccg ggc tcg   384
Gly Gly Gln Glu Asn Met Ser Ala Ala Pro His Val Leu Pro Gly Ser
        115                 120                 125
cgc gat ggt ttc cgc atg ggc gat gcc aag ctg gtc gac acc atg atc   432
Arg Asp Gly Phe Arg Met Gly Asp Ala Lys Leu Val Asp Thr Met Ile
    130                 135                 140
gtc gac ggc ctg tgg gac gtg tac aac cag tac cac atg ggc atc acc   480
Val Asp Gly Leu Trp Asp Val Tyr Asn Gln Tyr His Met Gly Ile Thr
145                 150                 155                 160
```

```
gcc gag aac gtg gcc aag gaa tac ggc atc aca cgc gag gcg cag gat   528
Ala Glu Asn Val Ala Lys Glu Tyr Gly Ile Thr Arg Glu Ala Gln Asp
                165                 170                 175
gag ttc gcc gtc ggc tcg cag aac aag gcc gaa gcc gcg cag aag gcc   576
Glu Phe Ala Val Gly Ser Gln Asn Lys Ala Glu Ala Ala Gln Lys Ala
            180                 185                 190
ggc aag ttt gac gaa gag atc gtc ccg gtg ctg atc ccg cag cgc aag   624
Gly Lys Phe Asp Glu Glu Ile Val Pro Val Leu Ile Pro Gln Arg Lys
        195                 200                 205
ggc gac ccg gtg gcc ttc aag acc gac gag ttc gtg cgc cag ggc gcc   672
Gly Asp Pro Val Ala Phe Lys Thr Asp Glu Phe Val Arg Gln Gly Ala
    210                 215                 220
acg ctg gac agc atg tcc ggc ctc aag ccc gcc ttc gac aag gcc ggc   720
Thr Leu Asp Ser Met Ser Gly Leu Lys Pro Ala Phe Asp Lys Ala Gly
225                 230                 235                 240
acg gtg acc gcg gcc aac gcc tcg ggc ctg aac gac ggc gcc gcc gcg   768
Thr Val Thr Ala Ala Asn Ala Ser Gly Leu Asn Asp Gly Ala Ala Ala
                245                 250                 255
gtg gtg gtg atg tcg gcg gcc aag gcc aag gaa ctg ggc ctg acc ccg   816
Val Val Val Met Ser Ala Ala Lys Ala Lys Glu Leu Gly Leu Thr Pro
            260                 265                 270
ctg gcc acg atc aag agc tat gcc aac gcc ggt gtc gat ccc aag gtg   864
Leu Ala Thr Ile Lys Ser Tyr Ala Asn Ala Gly Val Asp Pro Lys Val
        275                 280                 285
atg ggc atg ggc ccg gtg ccg gcc tcc aag cgc gcc ctg tcg cgc gcc   912
Met Gly Met Gly Pro Val Pro Ala Ser Lys Arg Ala Leu Ser Arg Ala
    290                 295                 300
gag tgg acc ccg caa gac ctg gac ctg atg gag atc aac gag gcc ttt   960
Glu Trp Thr Pro Gln Asp Leu Asp Leu Met Glu Ile Asn Glu Ala Phe
```

```
305                 310                 315                 320
gcc gcg cag gcg ctg gcg gtg cac cag cag atg ggc tgg gac acc tcc   1008
Ala Ala Gln Ala Leu Ala Val His Gln Gln Met Gly Trp Asp Thr Ser
                325                 330                 335
aag gtc aat gtg aac ggc ggc gcc atc gcc atc ggc cac ccg atc ggc   1056
Lys Val Asn Val Asn Gly Gly Ala Ile Ala Ile Gly His Pro Ile Gly
            340                 345                 350
gcg tcg ggc tgc cgt atc ctg gtg acg ctg ctg cac gag atg aag cgc   1104
Ala Ser Gly Cys Arg Ile Leu Val Thr Leu Leu His Glu Met Lys Arg
        355                 360                 365
cgt gac gcg aag aag ggc ctg gcc tcg ctg tgc atc ggc ggc ggc atg   1152
Arg Asp Ala Lys Lys Gly Leu Ala Ser Leu Cys Ile Gly Gly Gly Met
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ggc gtg gcg ctg gca gtc gag cgc aaa                               1179
Gly Val Ala Leu Ala Val Glu Arg Lys
385                 390
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<211> 393

<212> PRT

<213> Ralstonia eutropha

<400> 6

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Phe Gly Gly Ser Leu Ala Lys Ile Pro Ala Pro Glu Leu Gly Ala Val
            20                  25                  30
Val Ile Lys Ala Ala Leu Glu Arg Ala Gly Val Lys Pro Glu Gln Val
        35                  40                  45
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Ser Glu Val Ile Met Gly Gln Val Leu Thr Ala Gly Ser Gly Gln Asn
50 55 60
Pro Ala Arg Gln Ala Ala Ile Lys Ala Gly Leu Pro Ala Met Val Pro
65 70 75 80
Ala Met Thr Ile Asn Lys Val Cys Gly Ser Gly Leu Lys Ala Val Met
85 90 95
Leu Ala Ala Asn Ala Ile Met Ala Gly Asp Ala Glu Ile Val Val Ala
100 105 110
Gly Gly Gln Glu Asn Met Ser Ala Ala Pro His Val Leu Pro Gly Ser
115 120 125
Arg Asp Gly Phe Arg Met Gly Asp Ala Lys Leu Val Asp Thr Met Ile
130 135 140
Val Asp Gly Leu Trp Asp Val Tyr Asn Gln Tyr His Met Gly Ile Thr
145 150 155 160
Ala Glu Asn Val Ala Lys Glu Tyr Gly Ile Thr Arg Glu Ala Gln Asp
165 170 175
Glu Phe Ala Val Gly Ser Gln Asn Lys Ala Glu Ala Ala Gln Lys Ala
180 185 190
Gly Lys Phe Asp Glu Glu Ile Val Pro Val Leu Ile Pro Gln Arg Lys
195 200 205
Gly Asp Pro Val Ala Phe Lys Thr Asp Glu Phe Val Arg Gln Gly Ala
210 215 220
Thr Leu Asp Ser Met Ser Gly Leu Lys Pro Ala Phe Asp Lys Ala Gly
225 230 235 240
Thr Val Thr Ala Ala Asn Ala Ser Gly Leu Asn Asp Gly Ala Ala Ala
245 250 255
Val Val Val Met Ser Ala Ala Lys Ala Lys Glu Leu Gly Leu Thr Pro
260 265 270
Leu Ala Thr Ile Lys Ser Tyr Ala Asn Ala Gly Val Asp Pro Lys Val

275 280 285

Met Gly Met Gly Pro Val Pro Ala Ser Lys Arg Ala Leu Ser Arg Ala

290 295 300

Glu Trp Thr Pro Gln Asp Leu Asp Leu Met Glu Ile Asn Glu Ala Phe

305 310 315 320

Ala Ala Gln Ala Leu Ala Val His Gln Gln Met Gly Trp Asp Thr Ser

325 330 335

Lys Val Asn Val Asn Gly Gly Ala Ile Ala Ile Gly His Pro Ile Gly

340 345 350

Ala Ser Gly Cys Arg Ile Leu Val Thr Leu Leu His Glu Met Lys Arg

355 360 365

Arg Asp Ala Lys Lys Gly Leu Ala Ser Leu Cys Ile Gly Gly Gly Met

370 375 380

Gly Val Ala Leu Ala Val Glu Arg Lys

385 390

<210> 7

<211> 738

<212> DNA

<213> Ralstonia eutropha

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Met Thr Gln Arg Ile Ala Tyr Val Thr Gly Gly Met Gly Gly Ile Gly

1 5 10 15

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acc gcc att tgc cag cgg ctg gcc aag gat ggc ttt cgt gtg gtg gcc   96
Thr Ala Ile Cys Gln Arg Leu Ala Lys Asp Gly Phe Arg Val Val Ala
                20                  25                  30

ggt tgc ggc ccc aac tcg ccg cgc cgc gaa aag tgg ctg gag cag cag   144
Gly Cys Gly Pro Asn Ser Pro Arg Arg Glu Lys Trp Leu Glu Gln Gln
            35                  40                  45

aag gcc ctg ggc ttc gat ttc att gcc tcg gaa ggc aat gtg gct gac   192
Lys Ala Leu Gly Phe Asp Phe Ile Ala Ser Glu Gly Asn Val Ala Asp
        50                  55                  60

tgg gac tcg acc aag acc gca ttc gac aag gtc aag tcc gag gtc ggc   240
Trp Asp Ser Thr Lys Thr Ala Phe Asp Lys Val Lys Ser Glu Val Gly
65                  70                  75                  80

gag gtt gat gtg ctg atc aac aac gcc ggt atc acc cgc gac gtg gtg   288
Glu Val Asp Val Leu Ile Asn Asn Ala Gly Ile Thr Arg Asp Val Val
                85                  90                  95

ttc cgc aag atg acc cgc gcc gac tgg gat gcg gtg atc gac acc aac   336
Phe Arg Lys Met Thr Arg Ala Asp Trp Asp Ala Val Ile Asp Thr Asn
            100                 105                 110

ctg acc tcg ctg ttc aac gtc acc aag cag gtg atc gac ggc atg gcc   384
Leu Thr Ser Leu Phe Asn Val Thr Lys Gln Val Ile Asp Gly Met Ala
        115                 120                 125

gac cgt ggc tgg ggc cgc atc gtc aac atc tcg tcg gtg aac ggg cag   432
Asp Arg Gly Trp Gly Arg Ile Val Asn Ile Ser Ser Val Asn Gly Gln
    130                 135                 140

aag ggc cag ttc ggc cag acc aac tac tcc acc gcc aag gcc ggc ctg   480
Lys Gly Gln Phe Gly Gln Thr Asn Tyr Ser Thr Ala Lys Ala Gly Leu
145                 150                 155                 160

cat ggc ttc acc atg gca ctg gcg cag gaa gtg gcg acc aag ggc gtg   528
His Gly Phe Thr Met Ala Leu Ala Gln Glu Val Ala Thr Lys Gly Val
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                  165                 170                 175
acc gtc aac acg gtc tct ccg ggc tat atc gcc acc gac atg gtc aag   576
Thr Val Asn Thr Val Ser Pro Gly Tyr Ile Ala Thr Asp Met Val Lys
              180                 185                 190
gcg atc cgc cag gac gtg ctc gac aag atc gtc gcg acg atc ccg gtc   624
Ala Ile Arg Gln Asp Val Leu Asp Lys Ile Val Ala Thr Ile Pro Val
         195                 200                 205
aag cgc ctg ggc ctg ccg gaa gag atc gcc tcg atc tgc gcc tgg ttg   672
Lys Arg Leu Gly Leu Pro Glu Glu Ile Ala Ser Ile Cys Ala Trp Leu
     210                 215                 220
tcg tcg gag gag tcc ggt ttc tcg acc ggc gcc gac ttc tcg ctc aac   720
Ser Ser Glu Glu Ser Gly Phe Ser Thr Gly Ala Asp Phe Ser Leu Asn
225                 230                 235                 240
ggc ggc ctg cat atg ggc                                       738
Gly Gly Leu His Met Gly
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<210> 8

<211> 246

<212> PRT

<213> Ralstonia eutropha

<400> 8

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Thr Ala Ile Cys Gln Arg Leu Ala Lys Asp Gly Phe Arg Val Val Ala
             20                  25                  30
Gly Cys Gly Pro Asn Ser Pro Arg Arg Glu Lys Trp Leu Glu Gln Gln
         35                  40                  45
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Lys Ala Leu Gly Phe Asp Phe Ile Ala Ser Glu Gly Asn Val Ala Asp
50 55 60
Trp Asp Ser Thr Lys Thr Ala Phe Asp Lys Val Lys Ser Glu Val Gly
65 70 75 80
Glu Val Asp Val Leu Ile Asn Asn Ala Gly Ile Thr Arg Asp Val Val
85 90 95
Phe Arg Lys Met Thr Arg Ala Asp Trp Asp Ala Val Ile Asp Thr Asn
100 105 110
Leu Thr Ser Leu Phe Asn Val Thr Lys Gln Val Ile Asp Gly Met Ala
115 120 125
Asp Arg Gly Trp Gly Arg Ile Val Asn Ile Ser Ser Val Asn Gly Gln
130 135 140
Lys Gly Gln Phe Gly Gln Thr Asn Tyr Ser Thr Ala Lys Ala Gly Leu
145 150 155 160
His Gly Phe Thr Met Ala Leu Ala Gln Glu Val Ala Thr Lys Gly Val
165 170 175
Thr Val Asn Thr Val Ser Pro Gly Tyr Ile Ala Thr Asp Met Val Lys
180 185 190
Ala Ile Arg Gln Asp Val Leu Asp Lys Ile Val Ala Thr Ile Pro Val
195 200 205
Lys Arg Leu Gly Leu Pro Glu Glu Ile Ala Ser Ile Cys Ala Trp Leu
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Ser Ser Glu Glu Ser Gly Phe Ser Thr Gly Ala Asp Phe Ser Leu Asn
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<210> 9

<211> 542

<212> DNA

<213> Pseudomonas sp. strain 61-3

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aaatgacct tcgacgacgg ccatgacagc ggcctgttca cctgggatta tctgtaccaa 180

ctggcacaac gtcaggaagc actttgggca gattatcttg cagaactcaa agcggctgga 240

aagtcccgcg acccaagcga atccatcgtc aagctgatgc tctaattcag gcctcttgct 300

ctttagaggg catttttctaa tttcatctgt ttgaatgctc cgctgtgcgg caagcgattg 360

gcctgcttgc gaaaaaaatt aaactcgggt aaccaatgga gctggcaagt tccctgcagt 420

gctctctgaa ctagaaagca acgttgtgca attaacggtc acccgagcag tagtacctgg 480

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ag 542

<210> 10

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<212> DNA

<213> Ralstonia eutropha

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ctatcggcgc gatggcgtga ccggccgcat ggccagcctg gtctggctgg cggactgagc 180

ccgccgctgc ctcactcgtc cttgcccctg gccgcctgcg cgcgctcggc ttcagccttg 240

cgtcggcggc ggccgggcgt gcccatgatg tagagcacca cgccaccggc gccatgccat 300

acatcaggaa ggtggcaacg cctgccacca cgttgtgctc ggtgatcgcc atcatcagcg 360

ccacgtagag ccagccaatg gccacgatgt acatcaaaaa ttcatccttc tcgcctatgc 420

tctggggcct cggcagatgc gagcgctgca taccgtccgg taggtcggga agcgtgcagt 480

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cctcccgctt tgggggaggc gcaagccggg tccattcgga tagcatctcc ccatgcaaag 780
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<211> 292
<212> DNA
<213> Ralstonia eutropha

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ggcccgcgaa tcgtttctgc ccgcgcggca ttcctcgctt tttgcgccaa ttcaccgggt 180
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gcagccagca ccatgttcgt gcagcgcggc cctcgcgggg gcgaggctgc ag 292